Case Docket No. P1614-8019
Date February 23, 1998

ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. §1.53(b) is the patent application of:
Inventor(s): Hansueli IMMER, Wolf-George FORSSMANN, Knut ADERMANN, Christian KLESSEN
For: PROCESS FOR PREPARING CARDIODILATIN FRAGMENTS; HIGHLY PURIFIED CARDIODILATIN FRAGMENTS AND INTERMEDIATE PRODUCTS FOR THE PREPARATION OF SAME

This application is a -- continuation X divisional -- continuation-in-part of Application No. 08/737,927.

XX Specification (43 pages)
XX 6 sheets of drawings
XX Declaration and Power of Attorney
X Copy from a prior application for continuation or divisional
XX Return Receipt Postcard
XX Prior application assigned of record to Boehringer Mannheim GMBH at reel 8418 and frame 0495
XX Please cancel claims 2-20 prior to calculation of the filing fee
XX A filing fee, calculated as shown below not including the above canceled claims:

	(Col. 1)	(Col. 2)	Small Entity			Other Than A Small Entity	
FOR:	No. Filed	No. Extra	RATE	FEE		RATE	FEE
BASIC FEE				$395	or		$790
TOTAL CLAIMS	1 - 20 =	* 0	× 11 =	--	or	× 22 =	
INDEP CLAIMS	1 - 3 =	* 0	× 41 =	--	or	× 82 =	
-- MULTIPLE DEPENDENT CLAIM PRESENTED			+135 =	--	or	+270 =	
			TOTAL	--	or		$790

* If the difference in Col. 1 is less than zero, enter "0" in Col. 2

Check No. 15619 in the amount of $ 790.00 is enclosed to cover the filing fee. The Commissioner is hereby authorized to charge payment for an additional filing fees associated with this communication or credit any overpayment to Deposit Account No. 14-1060.

Respectfully submitted,

NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

By: Monica Chin Kitts
Reg. No. 36,105

Metropolitan Square
655 Fifteenth Street, N.W.
Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000
MCK:sap

other possible additions:

XX Incorporation by reference (useable if continuation or divisional box is checked)
The disclosure of the prior application, from which a copy of the declaration is supplied as noted above is considered as being a part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

= English Translation Document

XX A Preliminary Amendment will be filed within two months.

= Small Entity Statement was filed in prior application, Status is still proper and desired.

XX An Information Disclosure Statement with PTO-1449.

= Nucleotide and/or Amino Acid Sequence Submission
- = Computer Readable Copy
- = Paper Copy (identical to computer copy)
- = Statement verifying identity of above copies

= Microfiche Computer Program (Appendix)

XX Priority of German application Serial No. P 44 20 381.1 filed on June 2, 1994 and P 195 13 784.1 filed on April 10, 1995 is claimed under 35 U.S.C. §119.

XX The certified copy has been filed in prior application Serial No. 08/737,927 filed December 2, 1996, and was acknowledged in the Office Action of August 28, 1997

4012/0A/WO

A Process for Preparing Cardiodilatin Fragments; Highly Purified cardiodilatin Fragments and Intermediate Products for the Preparation of Same

The invention relates to a process for the preparation of cardiodilatin fragments, to highly purified cardiodilatin fragments, and to appropriate intermediates for the preparation of said fragments.

The present invention is directed to a process for the preparation of cardiodilatin fragments of formula I

$$R^1\text{-ANP(105-121)-}R^2 \qquad (I),$$

having a chain length of 17 - 37 amino acids in total, wherein ANP(105-121) represents the amino acid sequence [SEQ ID NO. 1],

R^1 represents an amino acid chain of sequence ANP(90-104) [SEQ ID NO. 2] or fragments thereof having a chain length of 0 - 15 amino acids, and

R^2 represents an amino acid chain of sequence ANP(122-126) [SEQ ID NO. 3] or fragments thereof having a chain length of 0 - 5 amino acids,

wherein synthesis is effected via condensation of at least three partial fragments, and condensation of the partial fragments to give the cardiodilatin fragments of formula I is carried out between the amino acid positions Gly^{108} and Arg^{109} and the amino acid positions Gly^{120} and Cys^{121}.

Cardiodilatin is a peptide of the class of natriuretic peptides. These peptides play an important role in regulating the balance of salts and water in the body. The prototype of natriuretic hormones is cardiodilatin, also referred to in literature as atrial natriuretic peptide

(CDD/ANP). The isolation of cardiodilatin and the preparation of biologically active fragments of cardiodilatin are known from US-PS 4,751,284 (cf., W.G. Forssmann et al., Klin. Wochenschr. 1986, 64 (Suppl. VI), 4-12). A review on isolation and characterization of cardiodilatin and fragments thereof, as well as their physiological properties has been published in Eur. J. Clin. Invest. 1986, 16; 439-451 (W.G. Forssmann). From EP 0,349,545, a specific cardiodilatin fragment having a chain length of 32 amino acids is known. Meanwhile, this fragment is also referred to in literature as urodilatin (INN: ularitide). Furthermore, US 5,354,900 (Suntory) describes a biologically active fragment having a chain length of 28 amino acids, known as α-hANP. Further biologically active cardiodilatin fragments or derivatives thereof have been described in EP 0,180,615. Therein, in particular, cardiodilatin fragments are described which begin with the amino acid position Arg^{102} at the N-terminus and end with the amino acid position Arg^{125} or Arg^{126} at the C-terminus. Instead of the designation cardiodilatin, the literature frequently uses the designation "atrial natriuretic peptide" (ANP). In the numbering of the sequences of the cardiodilatin amino acids used in the following, reference is made to the nomenclature used for the ANF/CDD (1-126) peptide (=ANP) in EP 0,349,545.

A common structural feature of all hitherto known biologically active cardiodilatin fragments is the formation of a disulfide bridge between the amino acids Cys^{105} and Cys^{121}, resulting in a stable ring of 17 amino acids. It is believed that the formation of this ring is substantially responsible for the biological activity of the cardiodilatin derivatives. At position Cys^{105}, the cardiodilatin fragments are substituted by an amino acid chain R^1 having a chain length of 0 - 15 amino acids, and at position Cys^{121} by a chain R^2 having a chain length of 0 - 5 amino acids. In the [SEQ ID NO. 1], the central region ANP(105-121) is presented in linearized form.

105 110
R^1-Cys Phe Gly Gly Arg Met -
|
Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys-R^2
115 120

The cardiodilatin fragment ANP(95-126), with the INN designation ularitide, is a particularly stable and biologically active human peptide, having diuretic activity and a relaxing effect on the smooth vascular muscles, which is formed of 32 amino acids and has the following sequence, wherein both the cysteine amino acids at positions 11 and 27 in the peptide are forming a disulfide bridge:

H-Thr-Ala-Pro-Arg-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH

Urodilatin is found in human urine. EP 0,349,545 describes a process for recovering urodilatin from urine using alginic acid, wherein the peptides adsorbed to alginic acid are eluted, the eluate is fractionated according to conventional purification methods, and the active fraction is recovered using a test based on the examination of the relaxing effect of urodilatin on the smooth muscles.

Furthermore, EP 0,349,545 describes a stepwise chemical synthesis of urodilatin using the Merrifield process (J. Am. Chem. Soc. 1963, 85; 2149-2156), at a solid phase according to the ABI standard program following the Boc strategy. In addition, this patent specification describes the preparation of urodilatin from the partial fragment ANP(99-126). This fragment is bound to a solid phase, and is reacted with a second partial fragment, the tetrapeptide Boc-Thr(But)-Ala-Pro-Arg(Tos). The peptide ANP(95-126) obtained from the condensation is removed from the support, subjected to cyclization after removal of the

protecting groups and subsequently, is processed and purified in a per se known manner.

Similarly, EP 0,180,615 describes the chemical synthesis using a solid support, wherein formation of the cardiodilatin fragments described therein is effected successively, starting from the C-terminus in direction of the N-terminus. Here, condensation via partial fragments is not described.

However, the cardiodilatin fragments prepared according to the procedures described in literature did not have the purity necessary for clinical studies and for the authorization as medicinal product because, due to the synthesis, peptide impurities had been introduced into the final product which could not be removed even by subsequent purification processes. Due to their immunogenic properties, the impurities may give rise to undesirable side-effects when administered to the patient, so that therapeutic application involved risk. Moreover, the synthesis could be accomplished at only a small scale under reasonable technical input and was not economically suitable for a larger production scale. Furthermore, another drawback of known processes for synthesis was the existing potential risk of racemization due to which the urodilatin was obtained with lower purity, lower biological activity and in insufficient yield. Racemization of the product which frequently occurs with existing syntheses often resulted in insufficient optical purity of the final product, and these impurities frequently cannot be removed or only with exceedingly high technical input.

Thus, it is an object of the invention to develop an improved process for the chemical synthesis of cardiodilatin fragments which does not involve the above-mentioned drawbacks.

The object of the invention is attained by performing the synthesis of cardiodilatin fragments on the basis of the Merrifield process using a specific selection of peptide fragments.

Surprisingly, the course of synthesis has been found to be optimal when the cardiodilatin fragments are formed using three partial fragments, with the condensation of the partial fragments to give the cardiodilatin fragment of formula I being performed in such fashion that the formation is effected via condensation of partial fragments and bond formation between the amino acid positions Gly^{108} and Arg^{109} and the amino acid positions Gly^{120} and Cys^{121}. This process is advantageous in that the cardiodilatin fragments of formula I can be obtained in higher yields and in higher purity as compared to the synthetic processes known from prior art.

The synthesis of the cardiodilatin fragments of formula I is effected in such way that initially, the three partial fragments having the sequences R^1-ANP(105-108), ANP(109-120) and ANP(121)-R^2 are prepared according to the Merrifield process. Then, preferably, condensation of the three partial fragments to give the cardiodilatin fragment of formula I is effected in two partial steps, whereby in a first step, condensation between the amino acid positions Gly^{120} and Cys^{121} of the partial fragments ANP(109-120) and Cys^{121}-R^2 is effected, with the intermediate fragment ANP(109-121)-R^2 being formed. Then, in a subsequent second step, condensation of the thus obtained fragment ANP(109-121)-R^2 with the third partial fragment R^1-ANP(105-108) is effected, forming the desired cardiodilatin fragment of formula I. Using the process according to the invention, the yield of cardiodilatin fragments is between 15 and 20%, based on the amount of each cardiodilatin partial fragment used as starting material.

The three partial fragments having the sequences R^1-ANP(105-108), ANP(109-120) and ANP(121)-R^2 are prepared according to the Merrifield process, wherein the amino acids with functional groups (hydroxy, carboxy, amino, or mercapto groups) present in the sequence are substituted by appropriate protecting groups. For example, as suitable protecting groups the following groups are possible:

protecting groups for hydroxy groups: Boc (t-butyloxycarbonyl), tBu (t-butyl ether);

protecting groups for amino functions: Fmoc (9-fluorenylmethoxycarbonyl), Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl), Trt (trityl);

protecting groups for carboxy groups: OtBu (t-butyl ester);

protecting groups for mercapto groups: Acm (acetamidomethyl) or Trt.

Here, the following protecting groups are preferred for the following amino acids: tBu for the amino acids Thr, Asn, Tyr or Ser; Pbf or Pmc for the amino acid Arg; Acm for the amino acid Cys; OtBu for the amino acid Asp; Trt for the amino acids Gln, Asn or Cys.

Using the Fmoc strategy (B. Riniker et al., Tetrahedron 1993, 49; 9307-9320), the protected partial fragments ANP(109-120), R^1-ANP(105-108) and ANP(121)-R^2 are formed on a solid support material. All the materials generally used in the Merrifield synthesis may serve as solid support materials. Preferred as support material is polystyrene functionalized as aminomethyl or benzhydrylamino compound. The superacid-sensitive bonding of the peptide fragments to the resin by means of the 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid linker allows their removal without impeding the side-chain protection. The fragments are purified by digestion with various solvents. Thus, the three starting fragments ANP(109-120), R^1-ANP(105-108) and ANP(121)-R^2 are obtained with a C-terminal free carboxyl

group and in good purity. When forming the peptides on the support resin, the yield in every single step of addition of one amino acid is nearly quantitative and is about 97-99%

The flow diagram in Figure 1 illustrates the principle of synthesis, with urodilatin ANP(95-126) as an example. Here, condensation of the fragment Boc-1-14-OH. (1) [this nomenclature corresponds to the general designation of fragment R^1-ANP(105-108), wherein R^1 = ANP(95-104)] with the fragment H-15-32-OtBu (5) [corresponding to an ANP nomenclature of ANP(109-121)-R^2, wherein R^2 = ANP(122-126)] is effected. This fragment (5) is synthesized from the fragments Fmoc-15-26-OH (2) [corresponding to an ANP nomenclature of ANP(109-120)] and H-27-32-OtBu (3c) [corresponding to an ANP nomenclature of ANP(121)-R^2]. Figure 2 represents the fragments synthesized and modified with protecting groups.

In the next step, the carboxyl group of fragment (3a) is converted to the t-butyl ester (3b) (cf., Riniker et al., 22nd Europ. Peptide Symposium Interlaken, September 1992 (L7)). Subsequent removal of the Fmoc group from fragment (3b) leads to the product (3c). This is fused with fragment (2), resulting in fragment (4). Removal of the Fmoc protecting group and condensation of the obtained fragment (5) with fragment (1) leads to the fully protected urodilatin (6). Removal of the protecting groups by treatment with trifluoroacetic acid and 1,3-propanedithiol as a scavenger provides the linear peptide (7) which is cyclized to crude urodilatin (8) by oxidation with iodine solution. This is desalted, purified and may be lyophilized subsequently. The synthesis of other cardiodilatin fragments is conducted in an analogous fashion.

The synthesis according to the invention, involving the described partial fragments ANP(109-120), R^1-ANP(105-

108) and ANP(121)-R^2 may be applied to all the cardiodilatin fragments of formula I. In particular, cardiodilatin fragments are possible, wherein R^1 has a chain length of 0-15 amino acids of the sequence ANP(90-104) or fragments thereof. Preferred for R^1 are chain lengths of 1-15 or 3-10 amino acids, particularly the sequences ANP(95-104), ANP(99-104) and ANP(102-104). In particular, the group R^2 represents a chain length of 1-5 amino acids of the sequence ANP(122-126) or fragments thereof. Preferably, however, the sequences ANP(122-126) and ANP(122-125) are possible for R^2.

Preferably, the cardiodilatin fragments ANP(95-126), ANP(99-126) and ANP(102-126) may be prepared according to the process of the invention. The cardiodilatin fragments prepared by means of the process of the invention, as well as the partial fragments required for condensation have high optical purity in the range of about 96-99.9%, particularly about 98-99%.

Similarly, the synthesis is suitable for all the other derivatives of cardiodilatin fragments wherein one or more amino acids in the sequence of human ANP are replaced by other amino acids. In this meaning, replacement of amino acids includes corresponding substitutions, deletions or insertions of amino acids. For example, single or multiple amino acids may be replaced by the corresponding D-amino acids (cf., EP 0,180,615). Likewise, peptides of similar structure and with a corresponding cyclic basic structure of 15-20 amino acids may be prepared in this way. Examples of such peptides are BNP (brain natriuretic peptide) or CNP (C-type natriuretic peptide). The structures of these peptides are described in J. Hypertension 1994, 12; 329-336 (N.C. Davidson and A.D. Struthers).

Likewise, the present invention is directed to novel partial fragments of ANP which are utilized for the

preparation of cardiodilatin fragments of formula I according to the process of the invention.

More specifically, corresponding peptide fragments are those of the type R^1-ANP(105-108), wherein R^1 represents an amino acid chain of sequence ANP(90-104) or fragments thereof having a chain length of 0-15 amino acids, as well as their derivatives modified by protecting groups. Here, in particular, R^1 has the above-mentioned meanings. Another novel peptide fragment is the fragment having the amino acid sequence ANP(109-120), as well as its derivatives modified by protecting groups, which is employed as a starting material in the condensation with the partial fragment ANP(121)-R^2. Likewise, the corresponding ANP(121)-R^2 type peptide fragments represent a novelty and a subject matter of the invention, wherein R^2 represents an amino acid chain of sequence ANP(122-126) or fragments thereof having a chain length of 0-5 amino acids, as well as their derivatives modified by protecting groups. In particular, R^2 has the previously mentioned meaning. In addition, the invention is directed to the intermediate ANP(109-121)-R^2 which is formed from the condensation reaction of the partial fragments ANP(109-120) and ANP(121)-R^2 effected in the first reaction step.

Furthermore, the present invention relates to a process for preparing high-purity cardiodilatin fragments of formula I. Conventional synthetic processes and subsequent purification procedures on cardiodilatin fragments suffered from the drawback that in many cases a peptide purity in a range of merely 97-98% could be achieved.

EP 0,349,545 describes a purity level of about 98% in the case of urodilatin; therein, the amount of urodilatin prepared was merely on a smaller laboratory scale in the range of a few milligrams. The purification procedure described in Example 5 therein is based on a chromatography

on a LH column (eluant: 1% AcOH, 1% TFEtOH) and subsequent chromatography on a TSK column (Fractogel TSK-HW 40), wherein an aqueous solution of 10% AcOH and 1% TFEtOH was used as the eluant. In a final purification step, purification using preparative HPLC is effected, without any further indications on the eluant being made. Within the scope of later experiments on the preparation of larger amounts of urodilatin in the range of a few grams for performing clinical tests, it was determined, however, that in spite of multiple purification steps, the synthesized material could not be purified beyond a purity level of more than 98%.

A comparable situation resulted in the case of cardiodilatin fragments described in EP 0,180,615. Therein, for example, the purification for fragment ANP(102-126) - in Example III.A.3 referred to as hANVP(127-151) - by chromatography on a type G25F Sephadex column is described, where 0.5 M AcOH was used as the eluant. In a subsequent purification step by means of ion exchange chromatography on CM Sepharose or CM Cellulose using a solvent gradient of 0.01 M NH_4OAc/300 mM NH_4OAc at pH 4.5, the peptide is obtained in a purity of about 97%. Likewise, this purity achieved is not satisfactory for the requirements in drug manufacturing.

Surprisingly, it has been found that high-purity cardiodilatin fragments of formula I can be prepared if the crude product is purified using a reversed-phase HPLC column, and the cardiodilatin fragment is eluted using a buffer system containing triethylammonium phosphate (TEAP) and acetonitrile in aqueous solution. Here, preferably, the pH value of the elution buffer is adjusted to a value of 2-5, more specifically, of 2-3. Preferably, a type C_{18} column, for example, Biotage module type filled with YMC C_{18} is used as the reversed-phase HPLC column. This column is equilibrated with triethylammonium phosphate buffer prior

to loading the cardiodilatin fragments to be purified. For example, a solution of 10-200 mM TEAP, preferably 50 mM TEAP, is employed as a suitable buffer solution. The amount of buffer for column equilibration depends on the column size and this, in turn, on the amount of peptide to be purified. According to experience, a column volume of 75 x 300 mm (diameter x length) is required to purify an amount of peptide of 3-8 g of crude peptide. In this case, about 300 ml of a 50 mM TEAP buffer solution is required for equilibration. Subsequently, a solution of the concentrated crude product of cardiodilatin fragment is applied. As a solvent, for example, 10% acetic acid is suitable. Thereafter, the peptide is eluted in a continuous gradient by continuous charging of eluant (mixture of an aqueous solution of 10-200 mM TEAP and acetonitrile at a volume ratio of 2:3; pH 2-5). Elution of peptide is particularly advantageous if a continuous gradient of eluant is applied, where 22-28% of solvent gradient is used for a period of 90 minutes, followed by 28% of solvent gradient for 10 minutes and, eventually, 28-40% of gradient for 20 minutes. Preferably, the flow rate is 100-200 ml/min, more specifically, about 140 ml/min. In the meaning of the purification process according to the invention, a buffer mixture of triethylammonium phosphate in water and acetonitrile at a mixing ratio of from 1:3 to 2:1 (v/v), more specifically of about 2:3 (v/v) is used as elution buffer. The pH value of the buffer solution is 2-5, preferably 2-3, and more specifically about 2.25. TEAP may be used at a concentration of 10-200 mM, preferably 20-100 mM, and more specifically, of about 50 mM. According to the invention, optimum separation is achieved in the reversed-phase HPLC by equilibrating the column using 50 mM TEAP, pH 2.25, and eluting the peptide with a buffer consisting of 50 mM TEAP, pH 2.25, and acetonitrile at a ratio of 2:3.

Conventional purification procedures using aqueous 0.1% trifluoroacetic acid (TFA), for example, are not capa-

ble of further separating the polar impurities contained in the crude products, as are revealed in Fig. 5 in the example of urodilatin (Fig. 6). In contrast, in the case of the eluants used according to the invention, there is significant separation of both impurities (see Fig. 5). Furthermore, use of the eluant according to the invention is advantageous in that the base line in the HPLC chromatogram takes an absolutely steady course, while in the case of TFA, a strong drift can be observed. In addition, use of TFA suffers from the drawback that a higher back pressure builds up on the HPLC column, which is not the case for the eluant according to the invention.

Using the process according to the invention, high-purity cardiodilatin fragments of formula I are obtained in a purity of at least 99% and preferably, of up to 99.9%. Optionally, the cardiodilatin fragments may subsequently be converted to their physiologically acceptable salts, such as the acetate or citrate salts. The cardiodilatin fragments obtained are substantially free of peptide impurities so that not only the reversed-phase HPLC exhibits a single peak but also the much more sensitive method of capillary electrophoresis (CE) provides a single migration peak. In the case of urodilatin, the latter shows a mass of 3505.9 ± 1 in the MS analysis, without byproducts being detected. It turned out that the use of capillary electrophoresis allows an excellent demonstration of the differences between cardiodilatin fragments obtained according to prior art and the cardiodilatin fragments according to the invention. Figure 3 illustrates the CE chromatogram of a urodilatin production batch produced according to prior art. Herein, it can be clearly seen that the product still contains impurities. In contrast, Figure 4 represents the CE chromatogram of a urodilatin production batch produced according to the process of the invention and purified correspondingly. It is clearly obvious that the product is sub-

stantially free of other peptide impurities and exhibits a single migration peak in the capillary electrophoresis.

Therefore, the invention is directed to high-purity cardiodilatin fragments of formula I which are remarkable in that they do not contain substantial peptide impurities detectable by capillary electrophoresis and MS analysis, and that the purity analysis using capillary electrophoresis exhibits a single migration peak.

Similarly, the purification procedure according to the invention is also suitable for the preparation of analogous high-purity peptide compounds such as, e.g., BNP (brain natriuretic peptide), CNP (C-type natriuretic peptide) or derivatives thereof. The cyclic structure of ANP is based on the oxidation of two cysteine residues within the amino acid sequence, forming a cyclic ring of 17 amino acids. Other peptides which also form the characteristic cyclic structure of 15-20 amino acids, particularly 17 amino acids, such as, e.g., BNP or CNP, may be converted to the high-purity forms in the same fashion using the purification procedure according to the invention.

In the following embodiments, the invention will be illustrated using the selected representative cardiodilatin fragments ANP(95-126), ANP(99-126) and ANP(102-126).

Example 1

General Procedures of Solid-Phase Synthesis According to the Merrifield Process

a) Solid-Phase synthesis on a support resin

Starting from the C-terminus of the peptide to be synthesized, the first amino acid (AA) protected by the Fmoc group at the N-terminal end, is bound to the support

resin (Fmoc-AA-OHMPB-support resin). With a standard batch of 6.66 mmoles, the Fmoc protecting group is subsequently removed by adding 100 ml of a solvent mixture of piperidine and N-methylpyrrolidine (1:4 v/v). Then, the resin suspension is stirred for 10 minutes, subsequently filtrated, and again, 100 ml of the piperidine and NMP solvent mixture is added. Then, the suspension is stirred for 10 minutes, filtrated and subsequently washed with NMP an isopropanol,. and completeness of the reaction is checked using the Kaiser test.

Thereafter, the next amino acid is coupled to the resin. Initially, 20 mmoles of a 0.5 M solution of diisopropylethanylamine (DIPEA) in NMP is added to the resin, then 2.5 mmoles of a 0.5 M solution of 1-hydroxybenzotriazole (OHBT) in NMP, followed by 10 mmoles of the amino acid to be coupled in 25 ml of NMP. Thereafter, 11 mmoles of a 0.25 M solution of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) in NMP is added and stirred for 10 minutes. Completeness of the reaction is checked using the Kaiser test. Subsequently, the resin is filtrated and washed with NMP.

This process is continued in the same way, until the peptide chain of desired chain length of amino acids is built up on the resin. When synthesis is complete, the resin is dried to constant weight at 40°C.

b) Removal of the protected peptides from the support resin

Each of 10 suction flasks is charged with 75 ml of methanol and 3 ml of pyridine. 50 g of the support resin prepared according to step a) is stirred 10 times with 250 ml of 1% TFA in dry methylene chloride for one minute on the suction funnel, and is filtrated directly into the

respective suction flask. These 10 filtrates are checked using thin layer chromatography. Fractions containing product are combined and evaporated to dryness. The residue is triturated with deionized water, and the crystalline residue is filtrated off and dried.

Example 2

Preparation of Fragment ANP(109-120)

Following the general procedures of Example 1, and starting from 273 g of Fmoc-Gly-OHMPB-support resin (corresponding to 130 mmoles), 170.3 g of the fully protected cardiodilatin fragment ANP(109-120) is obtained.

Example 3

Preparation of Fragment ANP(121-126)

Following the general procedures of Example 1, and starting from 264 g of Fmoc-Tyr-OHMPB-support resin (corresponding to 115 mmoles), 150.7 g of the fully protected cardiodilatin fragment ANP(121-126) is obtained. Here, the N-terminal end of the fragment is protected by the Fmoc group.

Subsequently, the terminal hydroxy group at the C-terminal end of the fragment is converted to the OtBu protecting group. For esterification, 149 g of the fully protected fragment is dissolved in 500 ml of trifluoroethanol and 4.1 l of chloroform. This is followed by addition of 141 ml of TBTA (t-butyl-2,2,2-trichloroacetimidate), and the solution is heated at reflux for one hour. After the reaction is completed, the solution is concentrated to give a crystalline-oily residue, 6.8 l of diisopropyl ether is added, and the suspension is stirred at room temperature for 14 hours. The product is filtrated off and dried to

constant weight. 136.7 g of fragment 3b indicated in Fig. 2 is obtained.

Subsequently, the Fmoc protecting group at the N-terminal end of the fragment is removed, and conversion to fragment 3c indicated in Fig. 2 is effected. To this end, a solution of fragment 3b (135.7 g) in 1.8 l of DMF and 74 ml of diethylamine is stirred at room temperature for 3 hours. The solution is evaporated to complete dryness in a vacuum. The residue is digested with 1.4 l of deionized water and filtrated off. The wet product is taken up in 3 l of MTBE (methyl t-butyl ether). The solution is extracted with a saturated NaCl solution (2 x 100 ml), and the organic phase is dried with sodium sulfate. The solution is concentrated to a volume of 500 ml. Following addition of 1.5 l of isopropyl ether, stirring for two hours is effected. The product is filtrated and dried. The yield is 104.6 g of fragment 3c indicated in Fig. 2.

Example 4

Preparation of Fragment ANP(121-125)

In an analogous manner as described in Example 3, starting from 264 g of Fmoc-Arg(Pbf)-OHMPB-support resin and following the procedure described, 115.1 g of cardiodilatin fragment ANP(121-125) is obtained.

Example 5

Preparation of Fragment ANP(95-108)

Following the general procedures of Example 1, and starting from 210 g of Fmoc-Gly-OHMPB-support resin, 151.5 g of the fully protected cardiodilatin fragment ANP(95-108) is obtained.

Example 6

Preparation of Fragment ANP(99-108)

Following the general procedures of Example 1, and starting from 190 g of Fmoc-Gly-OHMPB-support resin, 145.1 g of the fully protected cardiodilatin fragment ANP(99-108) is obtained.

Example 7

Preparation of Fragment ANP(102-108)

Following the general procedures of Example 1, and starting from 220 g of Fmoc-Gly-OHMPB-support resin, 165.3 g of the fully protected cardiodilatin fragment ANP(102-108) is obtained.

Example 8

Condensation of the Partial Fragments to the Intermediate Product

The fragment ANP(109-120) is converted to the intermediate ANP(109-121)-R^2 by condensation with the C-terminal fragment ANP(121)-R^2 according to the following general process:

The fragment ANP(109-120), the amino terminus of which is protected by the Fmoc group, is dissolved in N-methylpyrrolidone. Subsequently, TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1-hydroxybenzotriazole and diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, the fragment ANP(121)-R^2 provided with an appropriate protecting group at the C-terminal end and dissolved in N-methylpyrrolidone is added to the solution. In the fol-

lowing, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with diisopropyl ether. Thereafter, the residue is suspended in acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated on a porcelain suction funnel, rewashed with acetonitrile and dried to constant weight in a vacuum chamber at 40°C. The thus obtained crude product represents the cardiodilatin fragment Fmoc-ANP(109-121)-R^2 protected at the amino terminus by the Fmoc protecting group. Thereafter, the Fmoc group is removed according to known procedures to obtain the intermediate product H-ANP(109-121)-R^2.

Example 9

Condensation of Fragments ANP(109-120) with ANP(121-126) to ANP(109-126)

Following the general procedure described in Example 8, 21.6 g of Fmoc-ANP(109-120) is dissolved in 650 ml of N-methylpyrrolidone. Subsequently, 3.2 g of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1.5 g of 1-hydroxybenzotriazole and 3.5 ml of diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, a solution of H-ANP(121-126)-OtBu, dissolved in 150 ml of N-methylpyrrolidone, is added. In the following, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto 4 l of diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with 500 ml of diisopropyl ether. There-

after, the residue is suspended in 600 ml of acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated on a porcelain suction funnel, rewashed with 300 ml of acetonitrile and dried to constant weight in a vacuum chamber at 40°C. Subsequently, the crude product Fmoc-ANP(109-126) thus obtained in an amount of 32.3 g is converted to the unprotected ANP(109-126) by addition of diethylamine. The yield is 30.2 g.

Example 10

Condensation of Fragments ANP(109-120) with ANP(121-125) to ANP(109-125)

Following the general procedure described in Example 8, 18.6 g of Fmoc-ANP(109-120) is dissolved in 600 ml of N-methylpyrrolidone. Subsequently, 3.0 g of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1.2 g of 1-hydroxybenzotriazole and 3.0 ml of diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, a solution of H-ANP(121-125)-OtBu, dissolved in 150 ml of N-methylpyrrolidone, is added. In the following, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto 4 l of diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with 450 ml of diisopropyl ether. Thereafter, the residue is suspended in 500 ml of acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated off on a porcelain suction funnel, rewashed with 250 ml of acetonitrile and dried to constant weight in a vacuum chamber at 40°C. Subsequently, the crude product Fmoc-ANP(109-125) thus obtained in an amount of 29.1 g is converted to the unprotected

ANP(109-125) by addition of diethylamine. The yield is 28.2 g.

Example 11

Condensation of the Partial Fragments to the Final Product

The intermediate ANP(109-121)-R^2 is converted to the final product R^1-ANP(105-121)-R^2 by condensation with the amino-terminal fragment R^1-ANP(105-108) according to the following general process:

The fragment R^1-ANP(105-108), the amino terminus of which is protected by an appropriate protecting group, is dissolved in N-methylpyrrolidone. Subsequently, TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1-hydroxybenzotriazole and diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, the fragment ANP(109-121)-R^2 provided with an appropriate protecting group at the C-terminal end and dissolved in N-methylpyrrolidone is added to the solution. In the following, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with diisopropyl ether. Thereafter, the residue is suspended in acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated off on a porcelain suction funnel, rewashed with acetonitrile and dried to constant weight in a vacuum chamber at 40°C. The thus obtained crude product represents the cardiodilatin fragment R^1-ANP(105-121)-R^2 protected by appropriate protecting groups at the amino terminus and the C-terminus. Thereafter, the protecting group is removed according to known procedures to obtain the intermediate product H-R^1-ANP(109-

121)-R^2. Following complete removal of the protecting groups, the obtained cardiodilatin fragment is converted to the cyclized derivative by oxidation and according to known procedures, for example, using iodine.

Example 12

Condensation of Fragments ANP(109-126) and ANP(95-108) to ANP(95-126)

a) Preparation of ANP(95-126)

Following the general procedure described in Example 11, 20.6 g of Boc-ANP(95-108) is dissolved in 400 ml of N-methylpyrrolidone. Subsequently, 2.7 g of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1.3 g of 1-hydroxybenzotriazole and 2.7 ml of diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, a solution of 29.4 g of H-ANP(109-126)-OtBu, dissolved in 400 ml of N-methylpyrrolidone, is added. In the following, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto 6.5 l of diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with 500 ml of diisopropyl ether. Thereafter, the residue is suspended in 600 ml of acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated off on a porcelain suction funnel, rewashed with 500 ml of acetonitrile and dried to constant weight in a vacuum chamber at 40°C. Subsequently, the crude product Boc-ANP(95-126)-OtBu thus obtained in an amount of 42.5 g is converted to the unprotected ANP(95-126) and dried. The yield is 27.5 g.

b) Cyclization of the deprotected linear ANP(95-126)

60 g of unprotected ANP(95-126) is dissolved in 16 l of 5% acetic acid in deionized water (v/v) and oxidized by addition of 570 ml of a 0.02 M methanolic iodine solution. The reaction is complete after 5 minutes. Excess iodine is destroyed by addition of a 0.1 M sodium thiosulfate solution. The cyclization solution obtained is subjected directly to further processing.

Example 13

Condensation of Fragments ANP(109-126) and ANP(99-108) to ANP(99-126)

Analogous to the procedure described in Example 12, 22.5 g of Boc-ANP(99-108) is dissolved in 400 ml of N-methylpyrrolidone. Subsequently, 2.9 g of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1.4 g of 1-hydroxybenzotriazole and 2.8 ml of diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, a solution of 30.6 g of H-ANP(109-126)-OtBu, dissolved in 400 ml of N-methylpyrrolidone, is added. In the following, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto 6.5 l of diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with 500 ml of diisopropyl ether. Thereafter, the residue is suspended in 600 ml of acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated off on a porcelain suction funnel, rewashed with 500 ml of acetonitrile and dried to constant weight in a vacuum chamber at 40°C. Subsequently, the crude product Boc-ANP(99-126)-OtBu thus

obtained in an amount of 44.7 g is converted to the unprotected ANP(99-126) and dried. The yield is 28.1 g.

Example 14

Condensation of Fragments ANP(109-126) and ANP(102-108) to ANP(102-126)

Analogous to the procedure described in Example 12, 20.4 g of Boc-ANP(102-108) is dissolved in 360 ml of N-methylpyrrolidone. Subsequently, 2.7 g of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), 1.4 g of 1-hydroxybenzotriazole and 2.6 ml of diisopropylethylamine are added to the solution at room temperature with stirring. Thereafter, a solution of 30.1 g of H-ANP(109-126)-OtBu, dissolved in 400 ml of N-methylpyrrolidone, is added. In the following, the reaction is monitored by thin layer chromatography. After about 2 hours, the reaction is complete. Then, the reaction mixture is dripped onto 6.5 l of diisopropyl ether with stirring and subsequently stirred for about 30 minutes. The precipitate is filtrated on a porcelain suction funnel over hard filter and washed twice with 500 ml of diisopropyl ether. Thereafter, the residue is suspended in 600 ml of acetonitrile and digested at room temperature with stirring. Subsequently, the product is filtrated off on a porcelain suction funnel, rewashed with 500 ml of acetonitrile and dried to constant weight in a vacuum chamber at 40°C. Subsequently, the crude product Boc-ANP(102-126)-OtBu thus obtained in an amount of 41.2 g is converted to the unprotected ANP(102-126) and dried. The yield is 26.9 g.

Example 15

Purification of ANP(95-126) and Preparation of the High-Purity Form

a) Concentrating the cyclized urodilatin [ANP(95-126)]

The cyclization solution (about 17 liters of 5% AcOH, in deionized water (v/v), contains about 60 g of cyclized urodilatin) is applied (flow rate 130 ml/min) on a glass column (diameter: 70 mm, length: 900 mm, filled with Vydac 218 TPB 2030) equilibrated with 1000 ml of buffer A3 (0.1% TFA (v/v) in deionized water).

Once application by pumping is finished, the peptide is eluted by continuous charging of buffer B3 (0.1% TFA in deionized water/ACN 2:3 v/v) in a continuous gradient (0% buffer B during 40 min; 15-35% buffer B during 90 min; 35% buffer B during 10 min; flow rate 130 ml/min).

urodilatin fractions showing a purity of more than 75% on monitoring by analytical HPLC are combined. These combined fractions are diluted with one volume equivalent of deionized water and applied (flow rate 140 ml/min) on a Biotage module (diameter: 75 mm, length: 300 mm, filled with YMC C_{18}, 120 A, 10 µm) equilibrated with 300 ml of buffer A3.

Subsequently, the concentrated peptide is eluted by washing the column with 100% buffer B3, and the acetonitrile is evaporated. The remaining solution is lyophilized.

Between 17 and 20 g of urodilatin with a purity of more than 90% is obtained.

b) Purification of the concentrated urodilatin

4.5 g of the concentrated urodilatin is dissolved in 250 ml of 10% AcOH in deionized water (v/v) and applied (flow rate 140 ml/min) on a Biotage module (diameter: 75 mm, length: 300 mm, filled with YMC C_{18}, 120 A, 10 µm) equilibrated with 300 ml of buffer A4 (50 mM TEAP, pH 2.25, in deionized water).

The peptide is eluted by continuous charging of buffer B4 (50 mM TEAP, pH 2.25 in deionized water/ACN 2:3 v/v) in a continuous gradient (22-28% B during 90 min; 28% B during 10 min; 28-40% B during 20 min; flow rate 140 ml/min).

urodilatin fractions showing a purity of more than 99% and impurities of not more than 0.5% on monitoring by analytical HPLC are combined. These combined fractions are diluted with one volume equivalent of deionized water and pumped onto the Biotage module previously cleaned with 1000 ml of buffer B3 and subsequently equilibrated with 300 ml of buffer A3. For desalting, a washing with 1200 ml of buffer A3 is made.

The pure product is eluted by washing the column with 1500 ml of buffer B3, and the acetonitrile is evaporated. The remaining solution is lyophilized.

The result is between 2.3 and 2.7 g of high-purity urodilatin.

c) Resalting of urodilatin x TFA to urodilatin acetate

2.5 g of high-purity urodilatin x TFA salt is dissolved in 80 ml of 5% AcOH, in deionized water v/v, and applied to a chromatography column (diameter:

20 mm, length: 300 mm, filled with 40 ml of Merck ion exchanger III acetate form) washed with 5% AcOH. A washing with 40 ml of 5% AcOH is made. The eluate, about 125 ml, is applied once more to the same ion exchange column. A washing with 55 ml of 5% AcOH is made. The eluate, about 180 ml, is filtrated clear over a polysulfone membrane (diameter 47 mm, 0.2 µm). The solution is lyophilized.

The result is between 2.05 and 2.30 g of high-purity urodilatin acetate.

Example 16

Purification of ANP(99-126) and Preparation of the High-Purity Form

a) Concentrating the cyclized cardiodilatin fragment ANP(99-126)

Analogous to Example 15a), the cyclization solution (about 15 liters of 5% AcOH, in deionized water (v/v), with a peptide content of about 50 g) is applied (flow rate 130 ml/min) on a glass column equilibrated with 1000 ml of buffer A3 (0.1% TFA (v/v) in deionized water). Once application by pumping is finished, the peptide is eluted by continuous charging of buffer B3 (0.1% TFA in deionized water/ACN 2:3 v/v) in a continuous gradient (0% buffer B during 40 min; 15-35% buffer B during 90 min; 35% buffer B during 10 min; flow rate 130 ml/min). Peptide fractions showing a purity of more than 75% on monitoring by analytical HPLC are combined. These combined fractions are diluted with one volume equivalent of deionized water and applied (flow rate 140 ml/min) on a Biotage module equilibrated with 300 ml of buffer A3. Subsequently, the concentrated peptide is eluted by washing the column with

100% buffer B3, and the acetonitrile is evaporated. The remaining solution is lyophilized.

The result is between 14 and 17 g of cardiodilatin fragment ANP(99-126) with a purity of more than 90%.

b) Purification of the concentrated ANP(99-126)

3.5 g of the cardiodilatin fragment concentrated according to Example 16a) is dissolved in 200 ml of 10% AcOH in deionized water (v/v) and applied (flow rate 140 ml/min) on a Biotage module equilibrated with 300 ml of buffer A4 (50 mM TEAP, pH 2.25, in deionized water). The peptide is eluted by continuous charging of buffer B4 (50 mM TEAP, pH 2.25 in deionized water/ACN 2:3 v/v) in a continuous gradient (22-28% B during 90 min; 28% B during 10 min; 28-40% B during 20 min; flow rate 140 ml/min).

Peptide fractions showing a purity of more than 99% and impurities of not more than 0.5% on monitoring by analytical HPLC are combined. These combined fractions are diluted with one volume equivalent of deionized water and pumped onto the Biotage module previously cleaned with 1000 ml of buffer B3 and subsequently equilibrated with 300 ml of buffer A3. For desalting, a washing with 1000 ml of buffer A3 is made.

The pure product is eluted by washing the column with 1500 ml of buffer B3, and the acetonitrile is evaporated. The remaining solution is lyophilized.

The result is between 1.7 and 2.2 g of high-purity cardiodilatin fragment ANP(99-126). Analogous to the procedure described in Example 14c), this fragment is converted to the corresponding acetate salt. The re-

sult is between 1.3 and 1.7 g of high-purity ANP(99-126) acetate.

Example 17

Purification of ANP(102-126) and Preparation of the High-Purity Form

a) Concentrating the cyclized cardiodilatin fragment ANP(102-126)

Analogous to Example 15a), the cyclization solution (about 18 liters of 5% AcOH, in deionized water (v/v), with a peptide content of about 65 g) is applied (flow rate 130 ml/min) on a glass column equilibrated with 1000 ml of buffer A3 (0.1% TFA (v/v) in deionized water). Once application by pumping is finished, the peptide is eluted by continuous charging of buffer B3 (0.1% TFA in deionized water/ACN 2:3 v/v) in a continuous gradient (0% buffer B during 40 min; 15-35% buffer B during 90 min; 35% buffer B during 10 min; flow rate 130 ml/min). Peptide fractions showing a purity of more than 75% on monitoring by analytical HPLC are combined. These combined fractions are diluted with one volume equivalent of deionized water and applied (flow rate 140 ml/min) on a Biotage module equilibrated with 300 ml of buffer A3. Subsequently, the concentrated peptide is eluted by washing the column with 100% buffer B3, and the acetonitrile is evaporated. The remaining solution is lyophilized.

The result is between 19 and 23 g of cardiodilatin fragment ANP(102-126) with a purity of more than 90%.

b) Purification of the concentrated ANP(102-126)

4.8 g of the cardiodilatin fragment concentrated according to Example 17a) is dissolved in 200 ml of 10% AcOH in deionized water (v/v) and applied (flow rate 140 ml/min) on a Biotage module equilibrated with 300 ml of buffer A4 (50 mM TEAP, pH 2.25, in deionized water). The peptide is eluted by continuous charging of buffer B4 (50 mM TEAP, pH 2.25 in deionized water/ACN 2:3 v/v) in a continuous gradient (22-28% B during 90 min; 28% B during 10 min; 28-40% B during 20 min; flow rate 140 ml/min).

Peptide fractions showing a purity of more than 99% and impurities of not more than 0.5% on monitoring by analytical HPLC are combined. These combined fractions are diluted with one volume equivalent of deionized water and pumped onto the Biotage module previously cleaned with 1000 ml of buffer B3 and subsequently equilibrated with 300 ml of buffer A3. For desalting, a washing with 1000 ml of buffer A3 is made.

The pure product is eluted by washing the column with 1500 ml of buffer B3, and the acetonitrile is evaporated. The remaining solution is lyophilized.

The result is between 1.9 and 2.4 g of high-purity cardiodilatin fragment ANP(102-126). Analogous to the procedure described in Example 14c), this fragment is converted to the corresponding acetate salt. The result is between 1.5 and 1.9 g of high-purity ANP(99-126) acetate.

Example 18

Analytical HPLC Examinations Using the ANP(95-126) Example

a) Elution with TEAP buffer, pH 2.25

50 μg of ANP(95-126) is injected onto an analytical HPLC column. A linear gradient of buffer B of 25-45% during 20 minutes (buffer A: 50 mM TEAP, pH 2.25; buffer B: mixture of A and acetonitrile at a volume ratio of 2:3) served as the eluant. The chromatogram in Fig. 5 reveals that two polar impurities are contained which may be separated by the eluant employed.

Legend to Fig. 5:
25 - 45 % in 20 min.
Buffer A: 50mM TEAP pH 2,25
Buffer B: A:ACN (2:3)
215 nm 1,0 ml/´C-Nr. 4040465 C
M+N 250/1/4°/3 Nuc 300 A5 u C18
D-2500
Method: 50 µg; TAG 243 CH:1; Peak reject: 5000
File: 1; Calculation method: area%; Table: 0; conc: area

No.	RT	Area	%	BC
5	7,82	53358	0,311	BV
6	8,08	84196	0,491	VV
7	9,07	386602	2,255	VV
8	9,78	1265799	7,384	VV
9	10,56	4701290	27,430	VV
10	10,92	10557085	61,582	VV
11	11,91	27613	0,161	TBB
12	12,82	8763	0,051	TBB
13	13,76	14346	0,084	BB
14	14,86	31959	0,186	BB
15	19,04	10892	0,064	BB
Total		17143003	100,00	

b) Elution with 0.1% TFA (trifluoroacetic acid)

Analogous to Example 18a), 50 µg of ANP(95-126) of the same production batch is applied onto an analytical HPLC column. A linear gradient of buffer B of 30-50% during 20 minutes (buffer A: 0.1% TFA in water; buffer B: mixture of A and acetonitrile at a volume ratio of 2:3) served as the eluant. The chromatogram in Fig. 6 reveals that separation of the contained impurities by means of this eluant is not effected. Compared to the chromatogram in Example a), the main peak is broader and the isolated product contains both of the polar impurities which can be recognized in the chromatogram of Fig. 5.

Legend to Fig. 6:

30 - 50 % B in 20 min.
Buffer A: 0,1 % TFA in water
Buffer B: A:ACN (2:3)
215 nm 1,0 ml/'C-Nr. 4011079 C
M+N 250/1/4°/3 Nuc 300 LA 5u C18
D-2500
Method: 50 µg; TAG 142; CH:1; Peak reject: 5000
File: 2; Calculation method: area%; Table: 0; conc: area

No.	RT	Area	%	BC
2	3,64	5073	0,040	BV
4	5,10	6624	0,053	BB
5	5,92	8161	0,065	BB
6	7,36	6814	0,054	BB
7	9,11	252878	2,012	BB
9	11,73	87629	0,697	BB
10	12,60	258273	2,055	BB
11	13,09	4578590	36,428	VV
12	13,26	7175177	57,086	VV
13	14,67	179155	1,425	TBB
14	17,48	10611	0,084	BB
Total		12568985	100,00	

Example 19

Purity Check by Capillary Electrophoresis

Lyophilized samples of the final products of cardiodilatin fragments from Examples 15 through 17 are dissolved in water at a concentration of 1 mg/ml and analyzed immediately. Capillary electrophoresis was performed using the Beckmann P/ACE 2100 system under the following conditions:

Capillary: Fused Silica by Supelco, separation length 50 cm, internal diameter 75 μm

Detection wave length: 200 nm

Injection period: 1 s

Separation buffer: 100 mM sodium phosphate, pH 2.5; 0.02% hydroxypropylmethylcellulose

Separation parameters: 25°C, 80 μA, 30 min

Figure 3 shows the chromatogram obtained for prior art urodilatin.

Figure 4 shows the chromatogram for high-purity urodilatin obtained according to Example 15.

A comparison of both chromatograms reveals that the urodilatin according to the invention differs significantly from prior art urodilatin. The urodilatin according to the invention is free of peptide impurities.

INDEX OF ABBREVIATIONS

Amino acids

Ala	L-Alanine
Asn	L-Asparagine
Asp	L-Asparaginic acid
Arg	L-Arginine
Cys	L-Cysteine
Gln	L-Glutamine
Gly	Glycine
Ile	L-Isoleucine
Leu	L-Leucine
Met	L-Methionine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine
Thr	L-Threonine
Tyr	L-Tyrosine

Protecting groups

Boc	t-Butyloxycarbonyl
Fmoc	9-Fluorenylmethoxycarbonyl
OtBu	t-Butyl ester
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulfonyl
tBu	t-Butyl ether
Acm	Acetamidomethyl
Trt	Trityl

Reagents/Solvents

ACN	Acetonitrile
TFA	Trifluoroacetic acid
TEAP	Triethylammonium phosphate

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Corange International Ltd.
(B) STREET: 36 Dover Street
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WIX 3RB

(A) NAME: Forssmann, Wolf-Georg, Prof. Dr. med. Dr. h.c.
(B) STREET: Bluecherstr. 5
(C) CITY: Hannover
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): 30175

(A) NAME: Adermann, Knut, Dr.
(B) STREET: Schleidenstr. 5
(C) CITY: Hannover
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): 30177

(A) NAME: Inuner, Hans-Ueli, Dr.
(B) STREET: Hasenweg 6
(C) CITY: Balsthal
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): 4710

(A) NAME: Klessen, Christian, Dr.
(B) STREET: Hauptstr. 26
(C) CITY: Lauterecken
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): 67742

(ii) TITLE OF INVENTION: A Process for Preparing Cardiodilatin Fragments; Highly Purified Cardiodilatin Fragments and Intermediate Products for the Preparation of Same

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE P 44 20 381.0

(B) FILING DATE: JUNE 02, 1994

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE 195 13 784.1

(B) FILING DATE: APRIL 10, 1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID No: 1:

Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
1 5 10 15

Cys

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Leu Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu Arg Arg Ser Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID No: 3:
Asn Ser Phe Arg Tyr
1 5

C L A I M S:

1. A process for the preparation of cardiodilatin fragments of formula I

$$R^1\text{-ANP(105-121)-}R^2 \qquad (I),$$

having a chain length of 17 - 37 amino acids in total, wherein ANP(105-121) represents the amino acid sequence [SEQ ID NO. 1],

R^1 represents an amino acid chain of sequence ANP(90-104) [SEQ ID NO. 2] or fragments thereof having a chain length of 0 - 15 amino acids, and

R^2 represents an amino acid chain of sequence ANP(122-126) [SEQ ID NO. 3] or fragments thereof having a chain length of 0 - 5 amino acids, characterized in that synthesis is effected via condensation of at least three partial fragments, the condensation of said partial fragments to give the cardiodilatin fragment of formula I being carried out between the amino acid positions Gly^{108} and Arg^{109} and the amino acid positions Gly^{120} and Cys^{121}.

2. The process according to claim 1, wherein

(a) in a first step, condensation of the partial fragments is effected between the amino acid positions Gly^{120} and Cys^{121} from the partial fragments ANP(109-120) and Cys^{121}-R^2, and

(b) in a second step, condensation of the partial fragments is effected between the amino acid positions Gly^{108} and Arg^{109} from the partial fragment ANP(109-121)-R^2 obtained according to step (a) and the partial fragment R^1-ANP(105-108).

3. The process according to one of claims 1 or 2, wherein R^2 represents the amino acid sequence ANP(122-126), characterized in that in a first step, the fragment ANP(109-126)-OtBu is prepared by condensation of the fragment Fmoc-ANP(109-120)-OH, which is synthesized on a solid support phase according to the Merrifield process and removed therefrom, with the fragment H-ANP(121-126)-OtBu, and subsequently, the Fmoc protecting group is removed from the resulting fragment Fmoc-ANP(109-126)-OtBu.

4. The process according to one of claims 1-3, wherein R^1 represents the amino acid sequence ANP(95-104), characterized in that the cardiodilatin fragment of formula I is prepared by condensation of the fragment Boc-ANP(95-108)-OH, which is synthesized on a solid support phase according to the Merrifield process and removed therefrom, with the fragment H-ANP(109-126)-OtBu, and subsequently, the protecting groups are removed from the resulting fragment Boc-ANP(95-126)-OtBu.

5. The process according to one of claims 1-4, characterized in that when forming the three partial fragments R^1-ANP(105-108), ANP(109-120) or ANP(121)-R^2 according to the Merrifield process, bonding to the solid support material is effected by means of a super-acid-sensitive linker.

6. The process according to one of claims 1-5, characterized in that the amino and hydroxy protecting groups are removed from the obtained fully protected cardiodilatin fragment R^1-ANP(105-121)-R^2, forming the fragment protected by the protecting group Acm at Cys^{105}, and subsequently, the protecting group Acm is

removed from the thus obtained fragment and thereafter, the cardiodilatin fragment is cyclized by oxidation.

7. The process according to one of claims 1-6, characterized in that R^1 represents the amino acid sequence selected from the group of ANP(95-104), ANP(99-104) and ANP(102-104).

8. The process according to one of claims 1-7, characterized in that R^2 represents the amino acid sequence selected from the group of ANP(122-125) and ANP(122-126).

9. A process for the preparation of high-purity cardiodilatin fragments R^1-ANP(105-121)-R^2 having a chain length of 17-37 amino acids in total, wherein R^1 represents an amino acid chain of sequence ANP(90-104) or fragments thereof having a chain length of 0-15 amino acids, and R^2 represents an amino acid chain of sequence ANP(122-126) or fragments thereof having a chain length of 0-5 amino acids, characterized in that purification of the crude product is performed using a reversed-phase HPLC column, and the cardiodilatin fragment is eluted with a buffer system containing triethylammonium phosphate and acetonitrile.

10. The process according to claim 9, characterized in that the elution is performed at a pH value of 2-5, more specifically of 2-3.

11. The process according to one of claims 9 or 10, characterized in that the reversed-phase HPLC column is equilibrated with a triethylammonium phosphate buffer, thereafter the concentrated crude product of the cardiodilatin fragment is applied and subsequently, the cardiodilatin fragment is eluted by continuous charging of a buffer mixture of triethylammonium phosphate in

water and acetonitrile (2:3 v/v) in a continuous gradient.

12. High-purity cardiodilatin fragments R^1-ANP(105-121)-R^2 having a chain length of 17-37 amino acids in total, wherein R^1 represents an amino acid chain of sequence ANP(90-104) or fragments thereof having a chain length of 0-15 amino acids, and R^2 represents an amino acid chain of sequence ANP(122-126) or fragments thereof having a chain length of 0-5 amino acids, characterized in that they are substantially free of peptide impurities and exhibit a single migration peak in the purity analysis using capillary electrophoresis.

13. The high-purity cardiodilatin fragments of claim 12, characterized in that R^1 represents an amino acid sequence selected from the group of ANP(95-104), ANP(99-104) and ANP(102-104).

14. The high-purity cardiodilatin fragments of claim 12 or 13, characterized in that R^2 represents an amino acid sequence selected from the group of ANP(122-125) and ANP(122-126).

15. The high-purity cardiodilatin fragments according to one of claims 12-14, selected from the group of ANP(95-126), ANP(99-126), ANP(102-126), and ANP(103-126).

16. Pharmaceutical formulations, containing the high-purity cardiodilatin fragment according to one of claims 12-15 in addition to physiologically acceptable adjuvants or additives.

17. Peptide fragments having the amino acid sequence R^1-ANP(105-108), wherein R^1 represents an amino acid chain of sequence ANP(90-104) or fragments thereof

having a chain length of 0-15 amino acids, as well as their derivatives modified by protecting groups.

18. Peptide fragment having the amino acid sequence ANP(109-120), as well as derivatives thereof modified by protecting groups.

19. Peptide fragments having the amino acid sequence ANP(109-121)-R^2, wherein R^2 represents an amino acid chain of sequence ANP(122-126) or fragments thereof having a chain length of 0-5 amino acids, as well as their derivatives modified by protecting groups.

20. Peptide fragments having the amino acid sequence Cys^{121}-R^2, wherein R^2 represents an amino acid chain of sequence ANP(122-126) or fragments thereof having a chain length of 3-5 amino acids, as well as their derivatives modified by protecting groups.

A B S T R A C T

The invention relates to a process for the preparation of cardiodilatin fragments, to highly purified cardiodilatin fragments, and to appropriate intermediates for the preparation of said fragments. Furthermore, the invention relates to highly purified cardiodilatin fragments which are free of peptide impurities and exhibit a single migration peak in capillary electrophoresis, as well as to appropriate processes for the preparation of same.

Fig. 1: Pathway of Synthesis

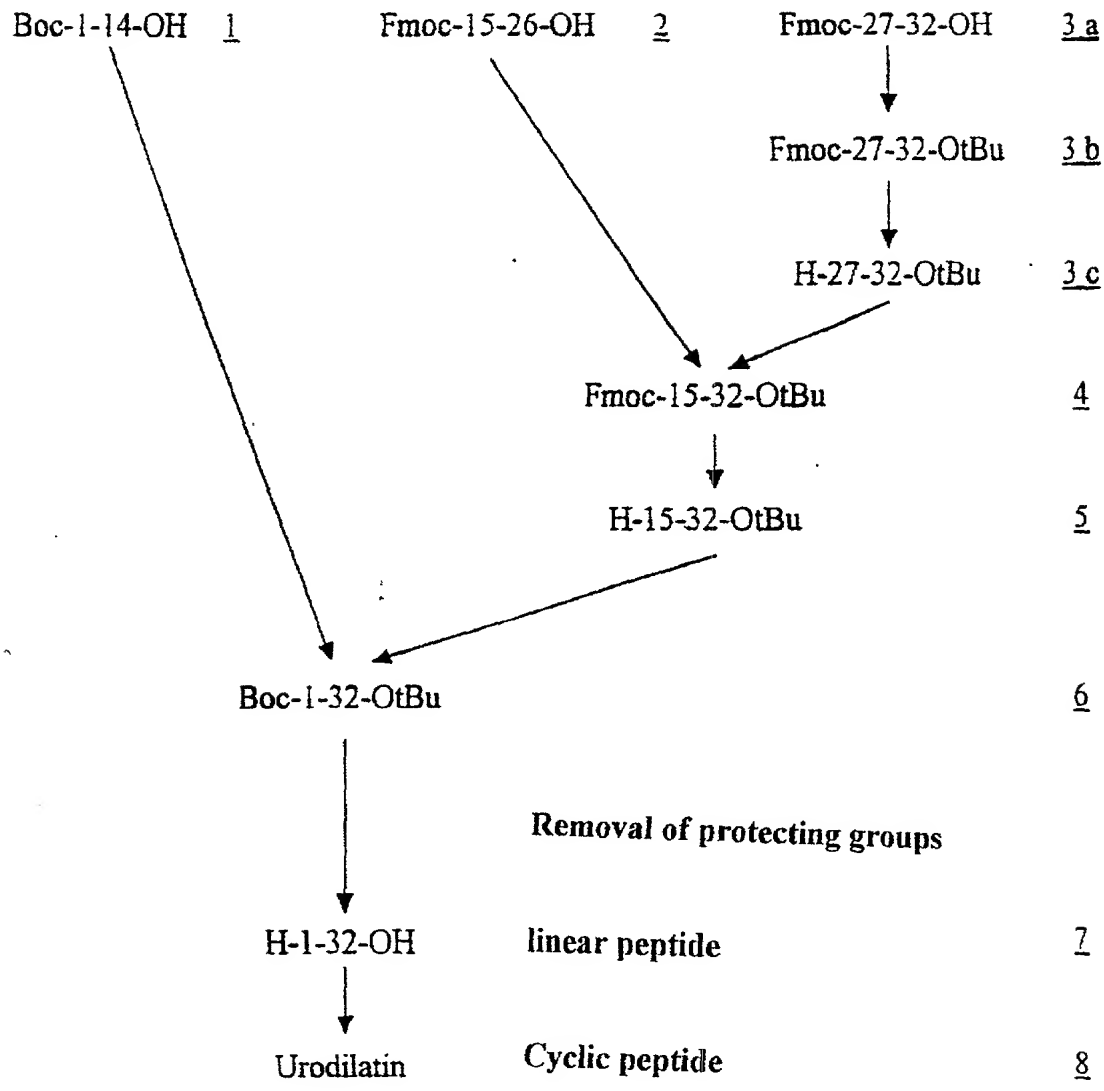
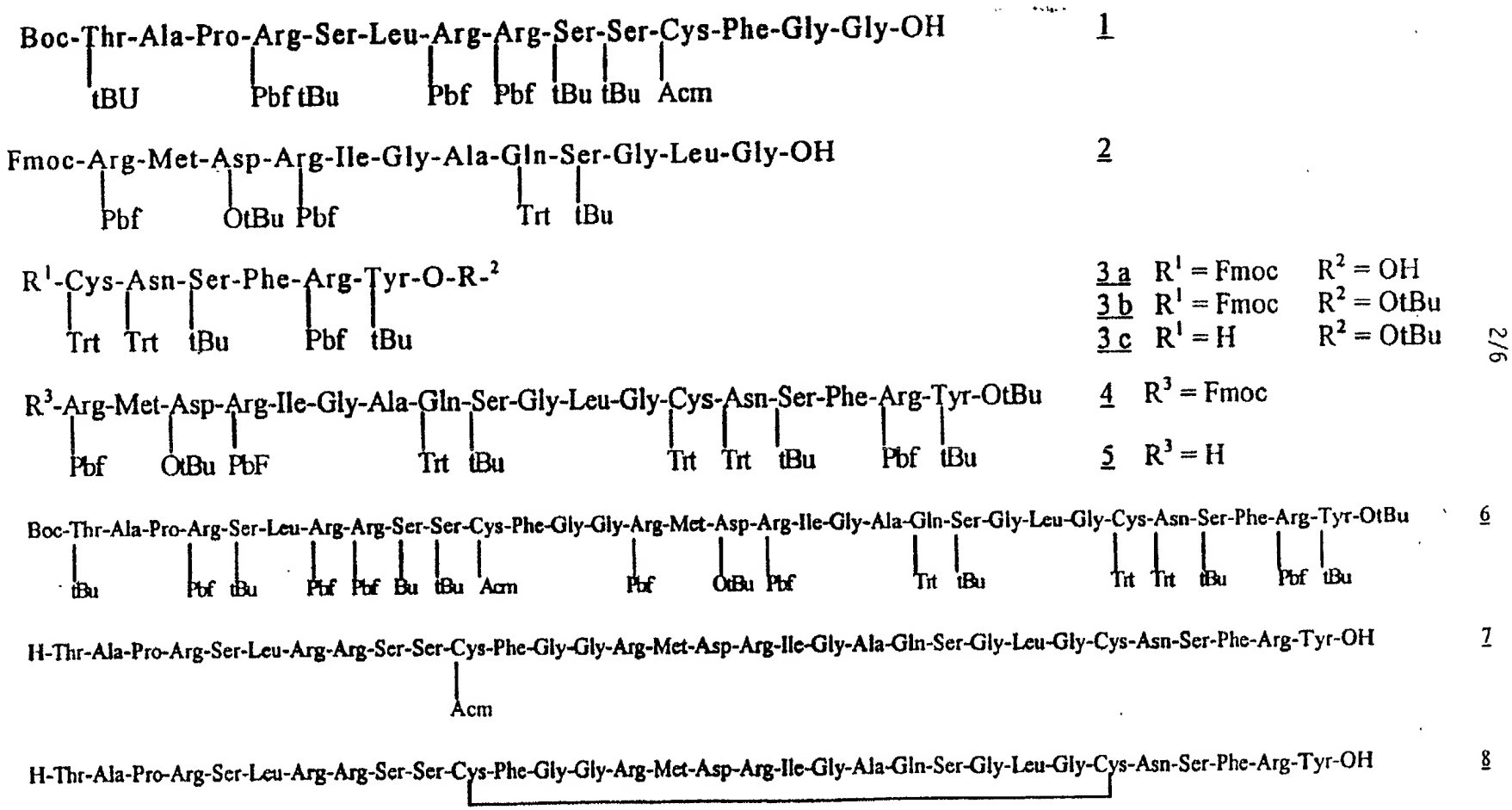


Fig. 2: Synthesized Fragments



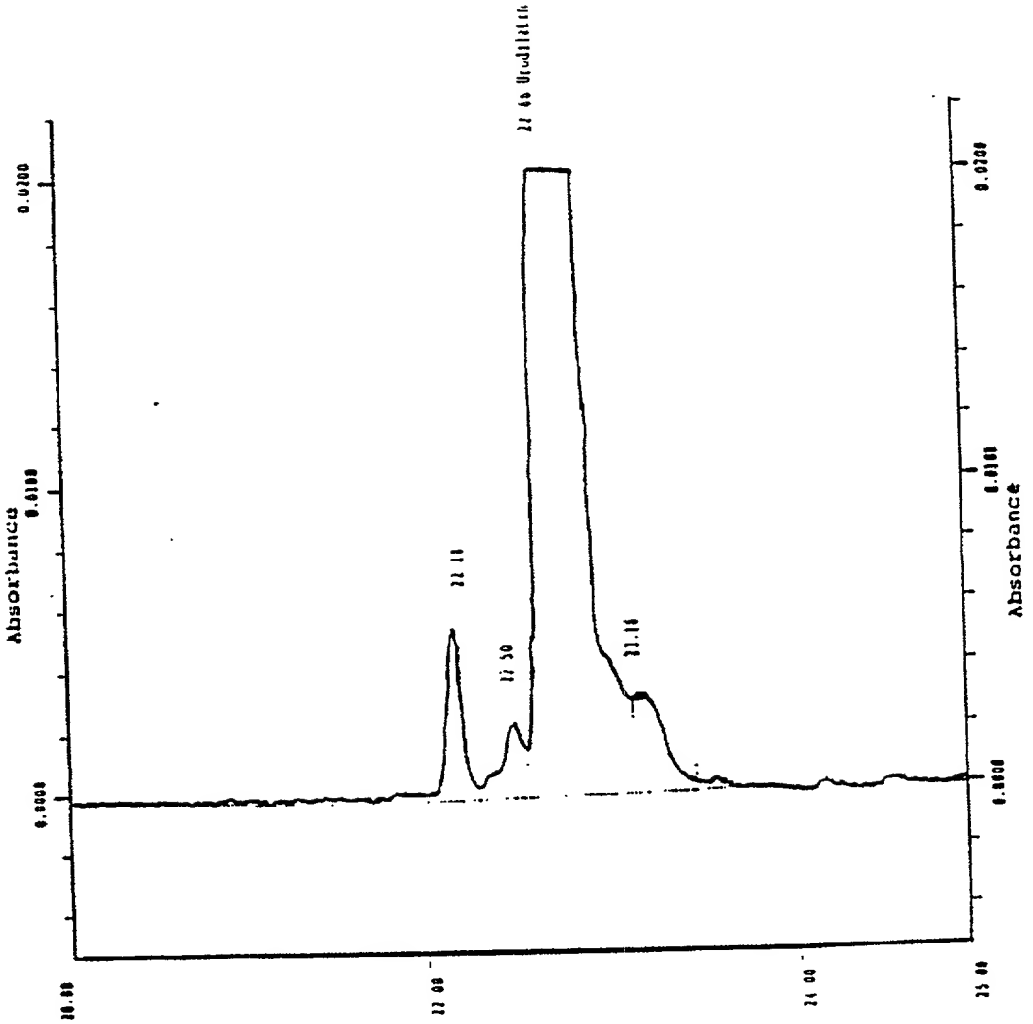


Fig. 3:

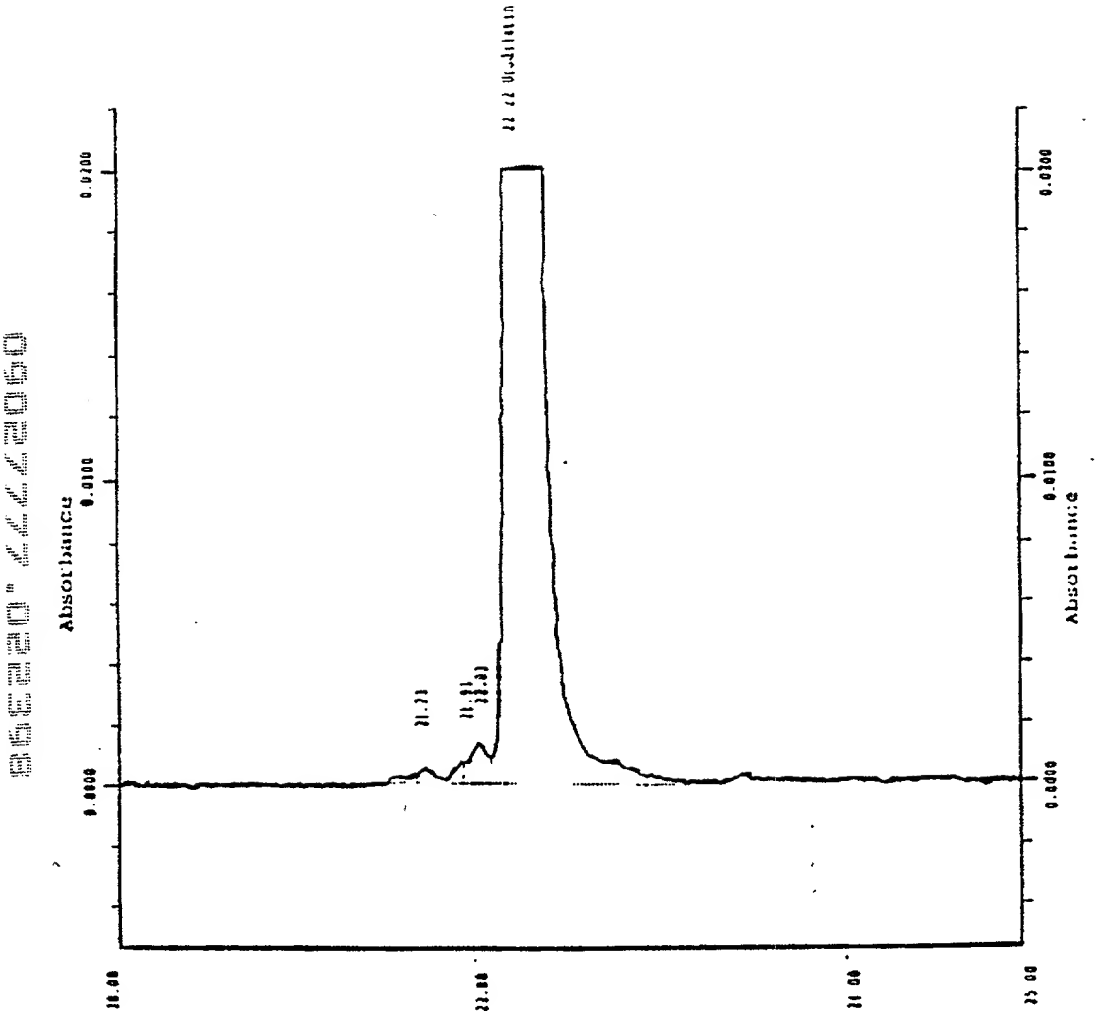


Fig. 4:

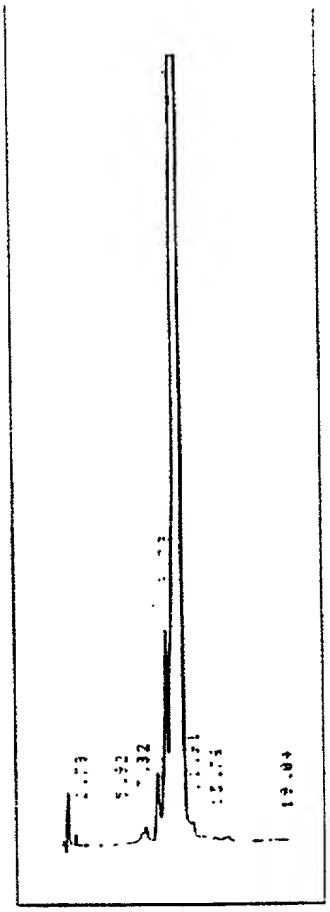


Fig. 5:

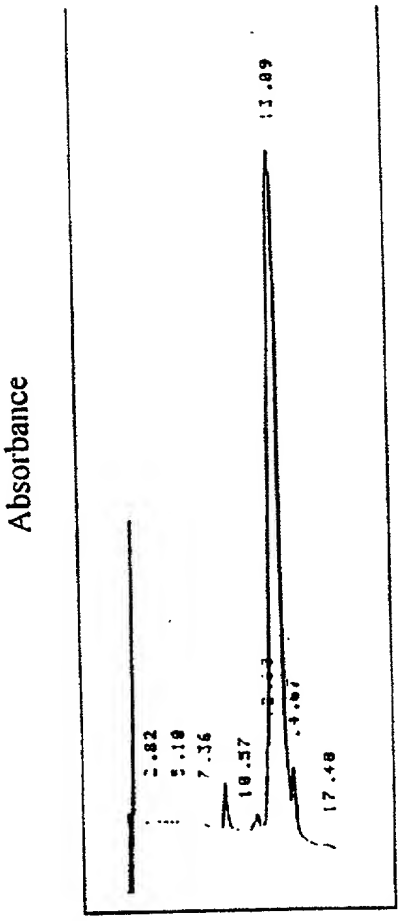


Fig. 6:

N, M, M & O Docket No. P1614-6052 NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled (Insert Title) PROCESS AND INTERMEDIATE PRODUCTS FOR PREPARING CARDIODILATIN FRAGMENTS, AND HIGHLY PURIFIED CARDIODILATIN FRAGMENTS

the specification of which is attached hereto unless the following box is checked:

☒ was filed on May 30, 1995 as United States Application Number or PCT International Application Number PCT/EP95/02050 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications. See note A on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
	P 44 20 381.0	DE	2/6/94	☒Yes ☐ No
	195 13 784.1	DE	10/4/95	☒Yes ☐ No
				☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Kitesner, Reg. No. 36,335; John R. Fuisz, Reg. No. 37,327; and Richard J. Berman, Reg. No. 39,107.

Please direct all communications to the following address:

NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor Hanspeli IMMER

Inventor's signature H. Immer (Immer) Date 22.11.96

Residence Hasenweg 6, CH-4710 Balsthal, Switzerland

Citizenship Switzerland

Post Office Address Same as above

Full name of second joint inventor, if any Wolf-Georg FORSSMANN

Inventor's signature _____ Date

Residence Blücherstrasse 5, D-30175 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of third joint inventor, if any Knut ADERMANN

Inventor's signature _____ Date

Residence Schleidenstrasse 5, D-30177 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fourth joint inventor, if any Christian KLESSEN

Inventor's signature _____ Date

Residence Hauptstrasse 26, D-67742 Lauterecken, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fifth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of sixth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of seventh joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of eighth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of ninth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

N, M, M & O Docket No. P1614-6052 NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled (Insert Title) PROCESS AND INTERMEDIATE PRODUCTS FOR PREPARING CARDIODILATIN FRAGMENTS, AND HIGHLY PURIFIED CARDIODILATIN FRAGMENTS the specification of which is attached hereto unless the following box is checked:

☒ was filed on May 30, 1995 as United States Application Number or PCT International Application Number PCT/EP95/02050 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
(List prior foreign applications. See note A on back of this page)	P 44 20 381.0	DE	2/6/94	☒Yes ☐ No
	195 13 784.1	DE	10/4/95	☒Yes ☐ No
				☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(List prior U.S. Applications or PCT International applications designating the U.S.)			
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Klesner, Reg. No. 36,335; John R. Fuisz, Reg. No. 37,327; and Richard J. Berman, Reg. No. 39,107.

Please direct all communications to the following address:

NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor Hansueli IMMER

Inventor's signature _____ Date _____

Residence Haseneweg 6, CH-4710 Balsthal, Switzerland

Citizenship Switzerland

Post Office Address Same as above

Full name of second joint inventor, if any Wolf-Georg FORSSMANN

Inventor's signature [signature] 21.11.96 Date

Residence Blücherstrasse 5, D-30175 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of third joint inventor, if any Knut ADERMANN

Inventor's signature ____ Date

Residence Schleidenstrasse 5, D-30177 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fourth joint inventor, if any Christian KLESSEN

Inventor's signature ____ Date

Residence Hauptstrasse 26, D-67742 Lauterecken, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fifth joint inventor, if any ____

Inventor's signature ____ Date

Residence ____

Citizenship ____

Post Office Address ____

Full name of sixth joint inventor, if any ____

Inventor's signature ____ Date

Residence ____

Citizenship ____

Post Office Address ____

Full name of seventh joint inventor, if any ____

Inventor's signature ____ Date

Residence ____

Citizenship ____

Post Office Address ____

Full name of eighth joint inventor, if any ____

Inventor's signature ____ Date

Residence ____

Citizenship ____

Post Office Address ____

Full name of ninth joint inventor, if any ____

Inventor's signature ____ Date

Residence ____

Citizenship ____

Post Office Address ____

N, M, M & O Docket No. P1614-6052 NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) PROCESS AND INTERMEDIATE PRODUCTS FOR PREPARING CARDIODILATIN FRAGMENTS, AND HIGHLY PURIFIED CARDIODILATIN FRAGMENTS
the specification of which is attached hereto unless the following box is checked:

☒ was filed on May 30, 1995 as United States Application Number or PCT International Application Number PCT/EP95/02050 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

				Priority Claimed
(List prior foreign applications. See note A on back of this page)	P 44 20 381.0 (Number)	DE (Country)	2/6/94 (Day/Month/Year Filed)	☒Yes ☐ No
	195 13 784.1 (Number)	DE (Country)	10/4/95 (Day/Month/Year Filed)	☒Yes ☐ No
	_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

_____ (Application Number)	_____ (Filing Date)
_____ (Application Number)	_____ (Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)
	_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Klesner, Reg. No. 36,335; John R. Fulsz, Reg. No. 37,327; and Richard J. Berman, Reg. No. 39,107.

Please direct all communications to the following address:

NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor Hansueli IMMER

Inventor's signature _____ Date _____

Residence Haseuweg 6, CH-4710 Balsthal, Switzerland

Citizenship Switzerland

Post Office Address Same as above

Full name of second joint inventor, if any Wolf-Georg FORSSMANN

Inventor's signature _____ Date

Residence Blücherstrasse 5, D-30175 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of third joint inventor, if any Knut ADERMANN

Inventor's signature K. Adermann 21.11.1996 Date

Residence Schleidenstrasse 5, D-30177 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fourth joint inventor, if any Christian KLESSEN

Inventor's signature _____ Date

Residence Hauptstrasse 26, D-67742 Lauterecken, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fifth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of sixth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of seventh joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of eighth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of ninth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

N, M, M & O Docket No. P1614-6052 NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled (Insert Title) PROCESS AND INTERMEDIATE PRODUCTS FOR PREPARING CARDIODILATIN FRAGMENTS, AND HIGHLY PURIFIED CARDIODILATIN FRAGMENTS the specification of which is attached hereto unless the following box is checked:

☒ was filed on May 30, 1995 as United States Application Number or PCT International Application Number PCT/EP95/02050 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications. See note A on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
	P 44 20 381.0	DE	2/6/94	☒Yes ☐ No
	195 13 784.1	DE	10/4/95	☒Yes ☐ No
				☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Kiesner, Reg. No. 36,335; John R. Pulsz, Reg. No. 37,327; and Richard J. Berman, Reg. No. 39,107.

Please direct all communications to the following address:

NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor Hansueli IMMER

Inventor's signature _____ Date

Residence Hasenweg 6, CH-4710 Balsthal, Switzerland

Citizenship Switzerland

Post Office Address Same as above

Full name of second joint inventor, if any Wolf-Georg FORSSMANN

Inventor's signature ______________________ Date ________

Residence Blücherstrasse 5, D-30175 Hannover, Federal Republic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of third joint inventor, if any Knut ADERMANN

Inventor's signature ______________________ Date ________

Residence Schleidenstrasse 5, D-30177 Hannover, Federal Repbulic of Garmany

Citizenship Germany

Post Office Address Same as above

Full name of fourth joint inventor, if any Christian KLESSEN

Inventor's signature Christian Klessen Date 11/27/96

Residence Hauptstrasse 26, D-67742 Lauterecken, Federal Repbulic of Germany

Citizenship Germany

Post Office Address Same as above

Full name of fifth joint inventor, if any ______________________

Inventor's signature ______________________ Date ________

Residence ______________________

Citizenship ______________________

Post Office Address ______________________

Full name of sixth joint inventor, if any ______________________

Inventor's signature ______________________ Date ________

Residence ______________________

Citizenship ______________________

Post Office Address ______________________

Full name of seventh joint inventor, if any ______________________

Inventor's signature ______________________ Date ________

Residence ______________________

Citizenship ______________________

Post Office Address ______________________

Full name of eighth joint inventor, if any ______________________

Inventor's signature ______________________ Date ________

Residence ______________________

Citizenship ______________________

Post Office Address ______________________

Full name of ninth joint inventor, if any ______________________

Inventor's signature ______________________ Date ________

Residence ______________________

Citizenship ______________________

Post Office Address ______________________